Stimulatory Effect of Lactic Acid Bacteria from Commercially Available Nozawana-zuke Pickle on Cytokine Expression by Mouse Spleen Cells

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We investigated the effect of lactic acid bacteria (LAB) isolated from eight samples of commercially available Nozawana-zuke, a traditional Japanese pickle, on cytokine expression by mouse spleen cell cultures. The 12 isolated strains of LAB (Nz1–Nz12), which were identified as genus *Lactobacillus* or *Leuconostoc* by the API50CHL test, enhanced the expression of interferon (IFN)-γ with 6 h of culture. Ten of these 12 LAB, particularly Nz8, enhanced interleukin (IL)-12 p40 expression. The actinase E- and Benzonase-treated or untreated cell wall fraction of Nz8 enhanced both IFN-γ and IL-12 p40 expression, while the cell plasma fraction had little effect. In the presence of anti-toll like receptor 4 antibody, the effect of the cell wall fraction of Nz8 was significantly abrogated. These results suggest that some LAB from Nozawana-zuke have a T helper 1-type immunoenhancing effect.

Key words: Nozawana-zuke; lactic acid bacteria; interferon-γ; interleukin-12; toll-like receptor 4

Nozawana-zuke is a traditional Japanese pickle made from Nozawana (mainly *Brassica campestris* L. var. *rapa*), a turnip green primarily grown in Shinshu. The pickle is manufactured by lactic acid fermentation after adding various inorganic salts and red pepper powder containing spicy components to Nozawana. The fermentation is mainly achieved by various plant-derived genera of lactic acid bacteria (LAB), including *Lactobacillus* and *Leuconostoc*. These LAB contribute to generating the sensory properties of Nozawana-zuke and preventing its contamination from disadvantageous bacteria by producing organic acids throughout both the manufacturing and storing periods.

LAB have been reported to have many beneficial effects on human health such as reducing cholesterol absorption, promoting lactose digestion, ameliorating gastrointestinal microflora, and producing some vitamins. In connection with these properties, the action of LAB for preventing cancer, viral infection, and allergic disorders has been reported, and sharp attention has consequently been paid to the immunomodulatory effect of LAB in recent years. Many studies have indicated that the mechanism for these useful effects of LAB is attributable to enhanced cell-mediated immunity. In fact, some LAB strains have been reported to enhance the production of T helper (Th)1-type cytokines, especially interferon (IFN)-γ and interleukin (IL)-12, and to skew the Th2 immune response toward a Th1 response in the immune system.

These useful strains of LAB, particularly defined as probiotics, have been applied as starter microorganisms for making functional foods. Many strains of LAB originating in human intestines and fermented milk products have actually been studied and utilized. However, the potential of LAB derived from plant-derived food materials has not been emphasized, except for a few reports.

We therefore separated LAB from commercially available Nozawana-zuke in this present study and characterized the bacteria according to their immunomodulatory effects. The effect of LAB on cytokine production in a mouse spleen cell culture was also investigated.

Materials and Methods

Isolation of bacteria. Eight samples of Nozawana-zuke were purchased from supermarkets in Nagano. LAB were isolated by the usual procedure. Briefly, 0.5 ml of diluted dipping sauce from a sample was mixed on culture plate with 9.5 ml of glucose, yeast extract, and peptone (GYP) medium containing 1.2% (w/v) of agar and 0.5% (w/v) CaCO₃. Sodium azide (0.01 mg/ml) and cycloheximide (0.01 mg/ml) were added as antibiotics. After an anaerobic incubation at 30 °C for 48–72 h, the colonies that had formed clear CaCO₃-melting zones around them were transferred to a
fresh GYP medium. The isolated strains were stored as frozen cultures in the same medium at –80 °C.

Classification of the bacteria. The isolated bacteria were classified with reference to 49 kinds of sugar fermentation pattern, using API50CH test strips and a 50CHL medium (BioMerieux, Maycy Y'Etoile, France). The cloned strains were cultivated overnight in 6 ml of the GYP medium at 30 °C. The cell suspension was transferred into API50CH strip wells and coated with paraffin oil. The strips were incubated at 30 °C. After 48 h of incubation, the carbohydrate fermentation by each strain was checked according to manufacturer’s recommendations. An additional test involving growth at 15 °C and 45 °C and gas production from glucose was also conducted.

Preparation of whole cells and cell components of N38. The bacteria cultured in the GYP medium at 30 °C for 24 h were harvested by centrifugation at 700 x g for 10 min. The precipitate was washed three times with sterile PBS, heated at 65 °C for 30 min, and used as heat-killed bacteria.

The cell component of the bacteria was fractionated by the method of Tejada-Simon and Pestka 18 with some modifications. Briefly, heat-killed bacteria were suspended in distilled water and disrupted by ultrasonic vibration at 40 kHz for 30 min with VS-50R equipment (Velcro-Clear, Tokyo, Japan). The sonicated sample was then heated at 60 °C for 15 min to inactivate the autolytic enzymes. The suspension was centrifuged at 800 x g for 30 min at 4 °C until the unbroken cells became visible by a microscopic examination. After further centrifugation at 12000 x g for 30 min, the supernatant was collected as the cell plasma fraction (CP). The precipitate after centrifugation was collected and washed three times with distilled water. The washed precipitate was suspended in a 50 mM Tris–HCl buffer (pH 7.5) and treated or not with 0.2 μg/ml of Actinase E (Kaken Pharmaceutical, Tokyo, Japan) and 12.5 U/ml of Benzonase® (Novagen, Madison, WI) at 37 °C for 6 h. The cell fraction respectively was collected as the enzyme-untreated cell wall fraction (UCW) and cell wall fraction (CW). All fractions were dialyzed against distilled water, lyophilized and kept at –80 °C until needed.

Preparation and culture of spleen cells. Male 6-week-old C3H/HeN mice were obtained from Japan SLC (Hamamatsu, Japan). The mice were killed by cervical dislocation, and their spleens were aseptically harvested. A single-cell suspension from the spleen was prepared by gently manipulating the tissues in an RPMI1640 medium (Nissui Pharmaceutical, Tokyo, Japan). The cells were washed twice with the medium and suspended for 5 min in a 17 mM Tris–HCl buffer (pH 7.2) containing 144 mM NH₄Cl. After centrifugation, the cells were resuspended at a concentration of 5 x 10⁶ viable cells/ml in the same medium containing 5% FBS (HyClone, Logan, UT), 100 IU/ml of penicillin, and 100 μg/ml of streptomycin. Cell cultures were set up with 2 ml of the cell suspension in quadruplicate on flat-bottomed microtiter plates (Falcon Labware, Oxnard, CA). The cell culture was maintained in a humidified atmosphere of 5% CO₂/95% air at 37 °C.

To evaluate the cytokine-enhancing activity of LAB, spleen cells were cultured for 6 h with or without 0.1 μg/ml of heat-killed bacteria. Similarly, spleen cells were cultured with 0.1 μg/ml of CP, UCW, or CW in order to evaluate the activity of the bacterial cell components. In addition, to reveal the involvement of TLR, 2.5 μg/ml of the anti-mouse toll-like receptor (TLR)2 rabbit antibody (Santa Cruz Biotechnology, Santa Cruz, CA) or the anti-mouse TLR4 rabbit antibody (Santa Cruz) was added before 1 h of the CW-added culture as described in the previous report. 19

Preparation of total RNA and the reverse transcription (RT)-polymerase chain reaction (PCR). Total RNA from spleen cells was extracted with the TRIzol® reagent (Invitrogen Life Technologies, Carlsbad, CA) according to the supplier’s recommendations. The reaction for RT was carried out by adding 1 μM each of dNTP, 2.5 U/μl of M-MLV reverse transcriptase (Invitrogen), and 10 pmol/μl of the Oligo d(T)₁₈ primer to 1 μg of total RNA, before incubating at 42 °C for 50 min. The resulting complementary DNA was subjected to PCR with a Taq PCR Core Kit (Qiagen, Chatsworth, CA) and 10 pmol/μl of primers for IFN-γ and glyceraldehyde-3-phosphate dehydrogenase (GAPDH). The primer sequences for amplifying the cytokines and GAPDH were as follows: interleukin IFN-γ (sense), 5'-AGC GGC TGA CTG AAC TCA GAT TGT AG-3'; IFN-γ (antisense), 5'-GTC ACA GTT TTC AGC TGT ATA GGG GTC-3'; IL-6 (sense), 5'-CTG GTG ACA ACC ACG GCC TTC CCT A-3'; IL-6 (antisense), 5'-ATG CTT AGG CAT AAC GCA CTA GGT T-3'; IL-10 (sense), GCT GAA GAC CTC TAT AGC GGG GTC-3'; IL-10 (antisense), CCC TGC AGC TCT CAA GTG TGG-3' (from base 420–440 and base 988–968, respectively; accession number M37897); IL-12 p40 (sense), GCG GTG CCT ATG TCT CAG CCT T-3'; IL-12 p40 (antisense), CAA GTT CTT GGG CGG GTG TG-3'; tumor necrosis factor (TNF)-α (sense), GCC GTG CCT ATG TCT CAG CCT T-3'; TNF-α (antisense), CAG AGT AAA GGG GTC AGA GTG-3' (from base 227–247 and
base 928–908, respectively; accession number M13049); GAPDH (sense), CCA CAG TCC ATG CCA TCA CTG-3'; and GAPDH (antisense), GGT CCA CCA CCC TGT TGC TGT AG-3' (from base 567–589 and 1019–997, respectively, accession number M32599). PCR was run for 24 cycles (for IFN-β/C13 and GAPDH) or 30 cycles (for IL-6, IL-10, IL-12 p40, and TNF-α/C11) of denaturation (94°C, 1 min), primer annealing (60°C, 1 min), and extension (72°C, 1 min) with a PTC-150 MiniCycler (MJ Research, Waltham, MA). Amplified DNA was subjected to gel electrophoresis on 2% agarose gel in a 0.04 M tris–acetate buffer (pH 8.0) containing 1 mM EDTA, and then visualized by ethidium bromide staining. The fluorescent intensity of the bands was digitalized with a Printgraph (Atto, Tokyo, Japan) and quantified with Densitograph Lane Analyzer software (Atto). Data for the cytokine expression were normalized to the expression of GAPDH.

Results

Classification of isolated LAB
Twelve strains of acid-producing bacteria were isolated from eight commercially available samples and were termed Nz1–Nz12. The result of the API50CHL test and of the additional test for classification of the strains is shown in Table 1. The growth temperature test showed that all strains other than Nz12 grew at 15°C, and not at 45°C. Moreover, only Nz2 and Nz3 produced gas from glucose. As a result, these 12 strains of LAB were classified as 10 strains of genus Lactobacillus (Nz1–Nz10) and 2 strains of genus Leuconostoc (Nz11 and Nz12).

Cytokine expression by the isolated LAB strains
The cytokine expression of mouse spleen cells cultured with or without each isolated strain is shown in Fig. 1. All strains enhanced IFN-β expression by up to 1.2–3.8-fold that under the LAB strain-free condition. Most of the strains, excepting Nz6, Nz9 and Nz12, also promoted IL-12 p40 expression by up to 1.2–4.4-folds. Although Nz8, the most effective enhancer of IFN-β and IL-12 p40 expression, enhanced the expression of IL-6 and TNF-α (by approximately 1.3- and 1.6-fold, respectively), the effect was statistically insignificant (Fig. 2). The expression of IL-10 was little influenced by Nz8.

<table>
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<tr>
<th>Isolated strain</th>
<th>Characteristic</th>
<th>API50CHL</th>
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<tr>
<td></td>
<td>Growth at $15^\circ$C &amp; $45^\circ$C</td>
<td>Gas production from glucose</td>
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<tr>
<td>Noz-1</td>
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<td>Noz-12</td>
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+, positive; –, negative

Fig. 1. Effect of LAB Strain on IFN-β and IL-12 p40 Expression.
The unshaded bar and shaded bar respectively represents the result of IFN-β and IL-12 p40 expression.
IFN-γ and IL-12 p40 expression by cell components of Nz8

The expression of IFN-γ and IL-12 p40 by spleen cells in the presence of CP, UCW, and CW from Nz8 is shown in Fig. 3. When the spleen cells were cultured with CP, no significant effect was apparent in either IFN-γ or IL-12 p40 expression. In contrast, the expression of these cytokine was markedly enhanced by UCW and CW. Treatment of CW with Actinase E and Benzonase had little influence on its cytokine-enhancing effects.

IFN-γ expression by CW from Nz8 in the presence of the anti-TLR antibody

The IFN-γ expression induced by CW from Nz8 in the presence of the anti-TLR2 or anti-TLR4 antibody is shown in Fig. 4. When spleen cells were treated with the anti-TLR4 antibody, the IFN-γ expression was significantly (P < 0.01) impaired, while the anti-TLR2 antibody had little influence on it. Moreover, no significant change in IFN-γ expression was apparent in the presence of all tested concentrations of PMB (data not shown).

Discussion

This study was undertaken to reveal the immunoregulatory effect of LAB from the plant-derived fermented food product, Nozawana-zuke. All the isolated strains shown in Table 1 were determined to be Gram-
positive in Gram staining and catalase-negative in the 
H₂O₂-hydrolyzing test (data not shown). As described 
previously,² a carbohydrate fermentation test, using the 
API50CHL identification kit, indicated that the isolated 
bacteria were of genus *Lactobacillus* or genus *Leuco-
nostoc* (Table 1).

Figure 1 shows the IFN-γ and IL-12 p40 expression 
by mouse spleen cells cultured with the isolated strains 
of LAB. In the presence of heat-killed LAB, almost all 
the strains enhanced the expression of cytokines, Nz8 
being the strongest expression inducer. We therefore 
further investigated the effect of Nz8 on the expression 
of the other cytokines.

As shown in Fig. 2, Nz8 enhanced IL-6 and TNF-α 
expression as well as that of IFN-γ and IL-12 p40 by the 
mouse spleen cell cultures. Nevertheless, only the 
expression of IFN-γ and IL-12 p40 was significant. 
Enhancement of the IL-12 p40 subunit reflects the 
production of biologically active IL-12 p70, because 
another subunit (p35) was constantly expressed whether 
the cells were stimulated or not.²³ IL-12 enhances the 
Th1-type immune response by inducing IFN-γ production.²⁶ 
Hence, these results suggest that Nz8 in 
prominently stimulated the Th1-type immune response.

In the challenge using the fractionated cell compo-
nents from Nz8, only UCW and CW exhibited IFN-γ-
and IL-12 p40-enhancing effects, while CP had no such 
an effect (Fig. 3). Moreover, the immunoenhancing 
effect of CW was insensitive to the Actinase E and 
Benzonase treatments. These results suggest that the 
immunoenhancing component of Nz8 was present 
mainly in the cell wall and that its effect was not 
attributable to either the protein or nucleic acid-based 
structure. In this respect, such cell wall components as a 
peptidoglycan and lipoteichoic acid from LAB have 
been reported to trigger cytokine production.¹⁸,²⁵ Cell 
wall components with a carbohydrate- or lipid-based 
structure such as peptidoglycan and lipoteichoic acid 
may therefore be related to the IL-12 p40- and IFN-γ-
enhancing activity of Nz8.

Previous studies have revealed the major cell wall 
component of Gram-positive bacteria to be the ligand of 
TLR2.²⁶,²⁷ However, the experiment using anti-TLR 
antibodies revealed the IFN-γ-enhancing effect of Nz8 
to be little affected by the presence of the anti-TLR2 
antibody. Not only was that, but the effect of Nz8 was 
abrogated by the anti-TLR4 antibody (Fig. 4). These 
results suggest that Nz8 was induced IFN-γ expression 
by spleen cells *via* the TLR4-signaling pathway. The 
most well-known ligand of TLR4 is LPS from Gram-
negative bacteria.²⁸ Therefore, we investigated whether 
the IFN-γ-enhancing effect was affected by PMB or not. 
As shown in Fig. 4, no significant change was observed 
in the expression of IFN-γ in the presence of PMB. 
PMB binds lipid A, a common essential structure of 
LPS, and disturbs the immunoenhancing activity of LPS 
in mouse spleen cell cultures.²⁹ These results suggest 
that the immunoenhancing effect of Nz8 found in this 
study was not due to the LPS being contaminated. A 
variety of endogenous ligands other than LPS such as 
fibronectin³⁰ and heat-shock proteins³¹–³³ have been 
reported to activate the TLR4-mediated signal pathway. 
These results may indicate that the specificity for the 
TLR4-mediated signal is not all that strict. Further 
investigation would reveal the relationship between the 
active component of Nz8 and TLR4.

In conclusion, we found that some LAB from 
Nozawana-zuke enhanced the Th1-type cytokine 
expression in a mouse spleen cell culture. These results 
suggest that some LAB from Nozawana-zuke had a Th1-
type immunoenhancing effect as well as the probiotic 
LAB strains separated from other materials.
References


